Involvement of Phosphatidylinositol-3 Kinase/AKT/PKCζ/λ Pathway in the Effect of Palmitate on Glucose-Induced Insulin Secretion

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Objectives: In the present study, a novel pathway by which palmitate potentiates glucose-induced insulin secretion by pancreatic beta cells was investigated.

Methods: Groups of freshly isolated islets were incubated in 10 mM glucose with palmitate, LY294002, wortmannin, and fumonisin B1 for measurement of insulin secretion by radioimmunoassay (RIA). Also, phosphorylation and content of AKT and PKC proteins were evaluated by immunoblotting.

Results: Glucose plus palmitate and glucose plus LY294002 or wortmannin (PI3K inhibitors) increased glucose-induced insulin secretion by isolated pancreatic islets. Glucose at 10 mM induced AKT and PKCζ/λ phosphorylation. Palmitate (0.1 mM) abolished glucose stimulation of AKT and PKCζ/λ phosphorylation possibly through PI3K inhibition because both LY294002 (50 μM) and wortmannin (100 nM) caused the same effect. The inhibitory effect of palmitate on glucose-induced AKT and PKCζ/λ phosphorylation and the stimulatory effect of palmitate on glucose-induced insulin secretion were not observed in the presence of fumonisin B1, an inhibitor of ceramide synthesis.

Conclusions: These findings support the proposition that palmitate increases insulin release in the presence of 10 mM glucose by inhibiting PI3K activity through a mechanism that involves ceramide synthesis.

Key Words: ceramide, insulin secretion, palmitate, pancreatic islets, PI3K signaling

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Glucose is the major stimulator of insulin secretion by pancreatic beta cell. Glucose enters pancreatic beta cell through the glucose transporter GLUT2,[1] and its metabolism leads to an increase of the ATP/ADP ratio, which closes the K_{ATP} channels and depolarizes cell membrane. This is followed by the opening of voltage-sensitive Ca^{2+} channels that rises intracellular calcium concentration ($[Ca^{2+}]_i$) and the exocytosis of insulin-containing granules.[2] Other nutrients, such as free fatty acids (FFA) and amino acids, modulate the process of glucose-induced insulin release.[3,4] Palmitate acutely does not affect insulin secretion under nonstimulatory glucose concentrations but markedly increases insulin release at high glucose concentrations.[5,6] This in vitro effect of palmitate is frequently correlated with in vivo observations that high plasma FFA levels are associated with hyperinsulinemia and insulin resistance, a key factor involved in the genesis of type II diabetes mellitus.[7,8] Long-chain fatty acids bind to G-protein–coupled receptor, GPR40, which is abundantly expressed in the pancreas,[9,10] and its activation potentiates the glucose effect on insulin secretion.[11] However, a recent study by Latour et al[12] has demonstrated that GPR40 activation accounts for only 50% of the full acute response to fatty acids by pancreatic islets. Therefore, additional mechanisms by which the palmitate increases glucose-induced insulin secretion remains to be fully elucidated.

The activation of pancreatic beta-cell insulin signaling pathway seems to play a key role in the control of insulin secretion due to an increase in $[Ca^{2+}]_i$.[13,14] Pancreatic beta cell from IR (βIRKO) and IGF1R (βIGFIRKO) knockout mice present low glucose-induced insulin secretion and insulin-dependent diabetes mellitus, probably due to impaired Ca^{2+}-activated exocytosis.[15] However, other studies have shown a negative feedback of insulin on its own secretion. This effect might be a result of PI3K activation[16] that leads to hyperpolarization of pancreatic beta-cell plasma membrane induced by the opening of the K_{ATP} channel.[17] We have recently demonstrated that under basal glucose concentration (5.6 mM), palmitate acutely up-regulates the insulin signaling pathway[18] and inhibits insulin secretion[6] in isolated rat pancreatic islets.

Glucose concentration determines the fate of palmitate metabolism in pancreatic beta cells.[6] High concentrations of glucose induce an increase of β-cell cytosolic malonyl-CoA content reducing therefore palmitate oxidation[19,20] and driving this fatty acid to ceramide synthesis.[21] Considering that palmitate is a substrate for de novo ceramide synthesis,[22] and synthetic ceramide analogs have been described to inhibit the PI3K/AKT pathway,[23,24] the aim of this study was to investigate the participation of the PI3K pathway in the mechanism by which palmitate increases insulin secretion under stimulatory glucose concentrations.

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MATERIALS AND METHODS

Reagents

Reagents for protein determination, the apparatus for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and nitrocellulose filters (0.45 μm) were from Bio-Rad (Melville, NY). Trizma, aprotinin, 1-4-dithiothreitol, phenylmethylsulfonyl fluoride, Triton X-100, glycerol, Tween 20, sodium palmitate, fumonisin B1, wortmannin, collagenase type V, and bovine serum albumin (fraction V) were obtained from Sigma Chemical Co (St Louis, Mo). Immunoblot enhanced chemiluminescence detection kit was purchased from Amersham Biosciences/GE Healthcare (Buckinghamshire, UK). Antibodies against phospho-AKT1/2/3 (Ser473), AKT1, AKT2, and PKC were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). Antibody against phospho-PKCζ/λ and LY294002 were acquired from Cell Signaling Technology, Inc (Beverly, Mass).

Animals

Female albino rats weighing 250 to 350 g were obtained from the Institute of Biomedical Sciences (University of São Paulo). The animals were kept under standard lighting conditions (12-hour light/dark cycle—lights on at 7:00 AM) at 23 ± 1°C, in groups of 5, and were allowed free access to standard rodent chow and water. The study was approved by the Ethical Committee of the Institute of Biomedical Sciences, Sao Paulo University.

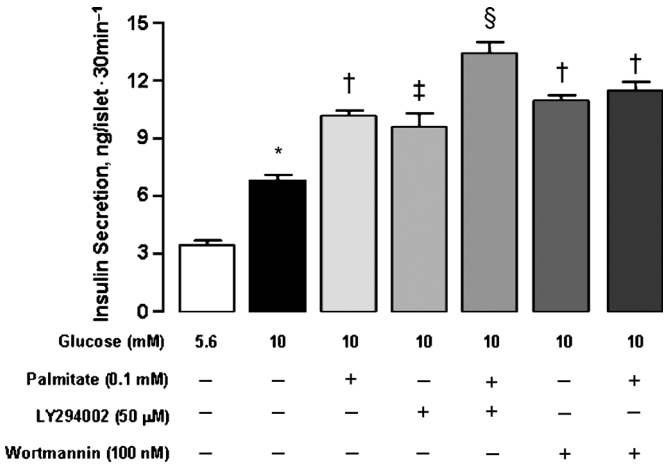

FIGURE 1. Insulin secretion from islets acutely incubated with PI3K inhibitors and palmitate. Groups of 5 freshly isolated islets were initially incubated for 30 minutes at 37°C in Krebs-Henseleit buffer containing 5.6 mM glucose. This solution was replaced, and islets were maintained for 30 minutes in Krebs-Henseleit buffer containing glucose at 5.6, 10, and 10 mM glucose plus palmitate, LY294002, and/or wortmannin. At the end of the incubation period, samples of the supernatants were collected for insulin measurement by RIA. The results are expressed as mean ± SEM (n = 12, *$P < 0.001$ vs 5.6 mM glucose, †$P < 0.05$ vs 10 mM glucose, ‡$P < 0.001$ vs 10 mM glucose, and §$P < 0.001$ vs 10 mM glucose plus palmitate).

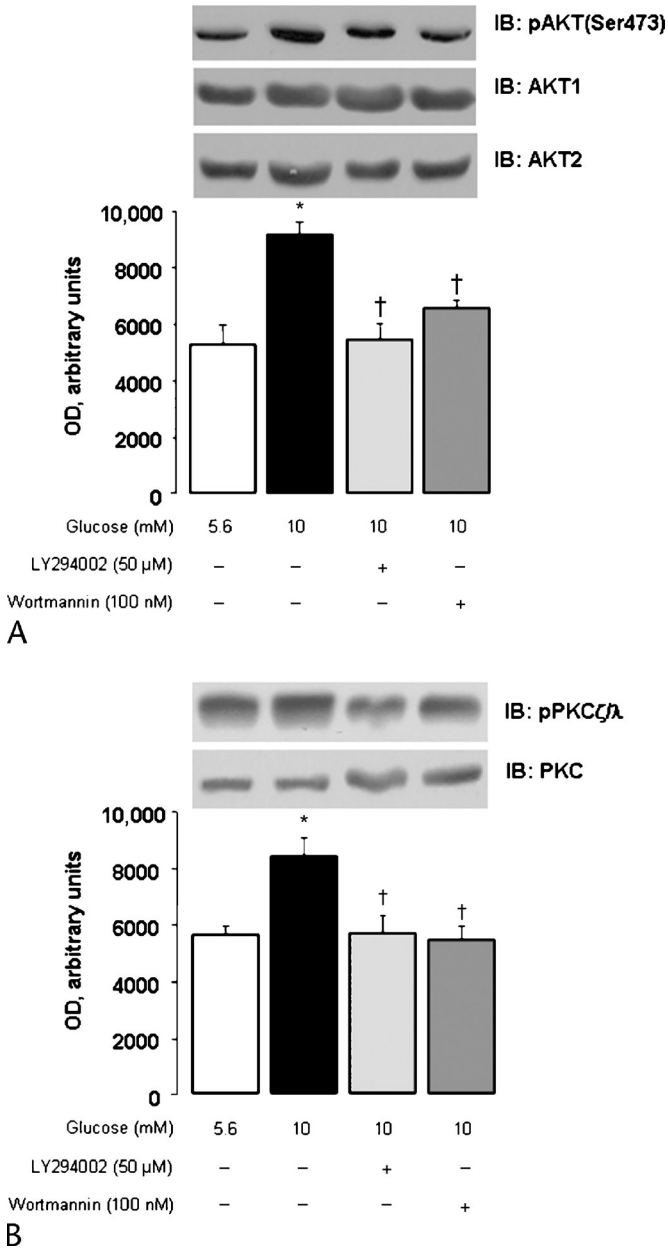

FIGURE 2. Effect of PI3K inhibitors on glucose-induced AKT (Ser473) and PKCζ/λ phosphorylation. Groups of 200 to 300 freshly islets were preincubated in the absence or presence of LY294002 or wortmannin. The islets were then incubated for 5 minutes (AKT) or 15 minutes (PKC), in Krebs-Henseleit buffer containing 5.6, 10, and 10 mM glucose plus LY294002 or 10 mM glucose plus wortmannin. Total protein extracts (100 μg) from homogenized islets were resolved in SDS-PAGE, transferred to nitrocellulose membranes and imunnoblotted (IB) with pAKT (Ser473), AKT1, and AKT2 antibodies (A) or PKC and pPKCζ/λ antibodies (B). Scanning densitometry was performed on autoradiograms from independent experiments. The results are expressed as mean ± SEM (n = 6, *$P < 0.05$ vs 5.6 mM glucose and †$P < 0.05$ vs glucose 10 mM). OD indicates optical density.

Isolation of Pancreatic Islets

The islets were isolated by collagenase digestion of the pancreas according to methodology previously described.[25] Hanks buffer containing 137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4.7H_2O$, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, and 4 mM $NaHCO_3$ was used for isolation and pooling the islets.

Insulin Secretion

Groups of 5 freshly isolated islets were initially incubated for 30 minutes at 37°C in 0.5 mL of Krebs–bicarbonate buffer (KB) (460 mM NaCl, 96 mM $NaHCO_3$, 20 mM KCl, 4 mM $MgCl_2$, and 4 mM $CaCl_2$) with 0.2% albumin. The buffer was equilibrated in a mixture of 95%:5% (O_2/CO_2) and pH was adjusted to 7.4. This solution was then replaced, and islets were maintained for additional 30 minutes in KB containing 5.6 mM glucose (G5.6), 10 mM glucose (G10), 10 mM glucose plus 0.1 mM palmitate (G10P), 10 mM glucose plus 50 μM LY294002, 10 mM glucose plus 100 nM wortmannin, or 10 mM glucose plus 15 μM fumonisin B1.

Before addition, palmitate was dissolved in ethanol to form an emulsified fatty-acid solution. The final ethanol concentration in the incubation medium was always lower than 0.5%. Preliminary experiments established that this concentration of ethanol has no effect on basal insulin release.[6] In experiments with LY294002, wortmannin, and fumonisin B1, the islets were preincubated and postincubated in the absence or in the presence of the inhibitor as described in the figure legends. These inhibitors were dissolved in dimethyl sulfoxide (DMSO) before addition to the incubation medium, and the final concentration of DMSO was lower than 0.1%. When required, both ethanol and DMSO were added in the controls (CTL) to a final concentration of 0.5% and 0.1%, respectively. At the end of the incubation period,

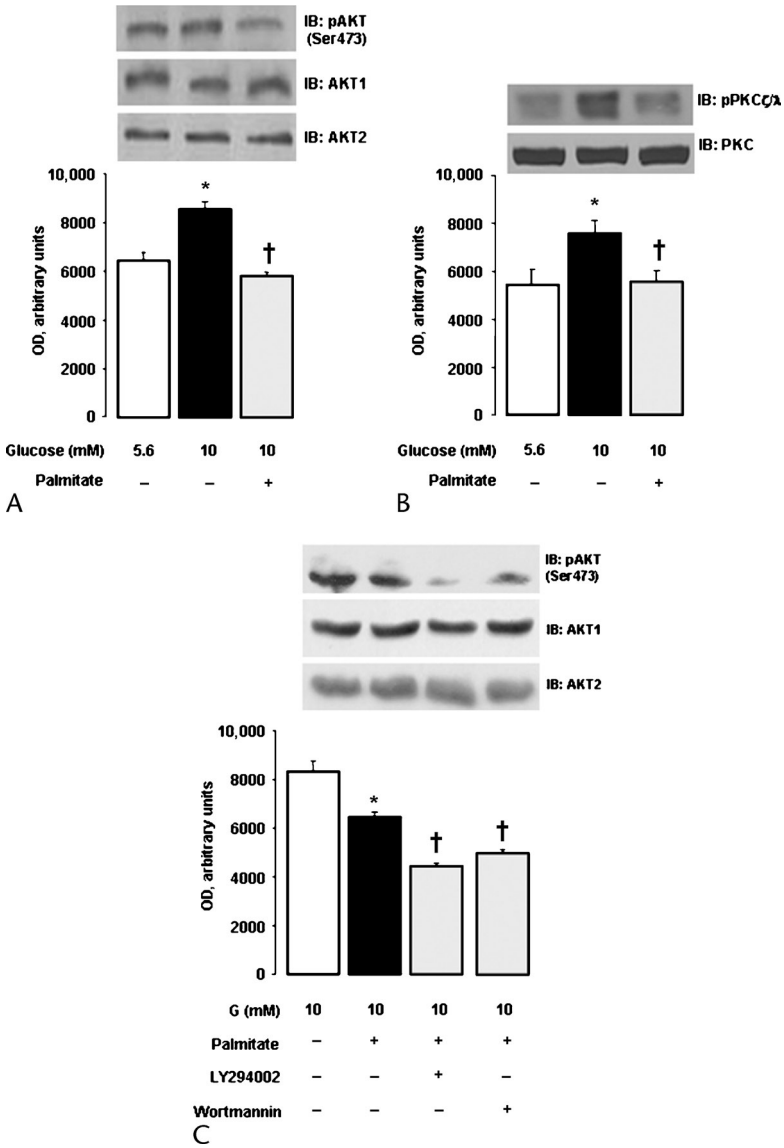

FIGURE 3. Effect of glucose and palmitate on AKT (Ser473) and PKCζ/λ phosphorylation. Groups of 200 to 300 freshly isolated islets were preincubated for 30 minutes at 37°C in Krebs-Henseleit buffer containing 5.6 mM glucose. This solution was replaced, and islets were maintained for 5 minutes (AKT) or 15 minutes (PKC) in Krebs-Henseleit buffer containing 5.6, 10, and 10 mM glucose plus palmitate, 10 mM glucose plus palmitate and wortmannin, or 10 mM glucose plus palmitate and LY294002. Protein (100 μg) extracts from homogenized islets were resolved in SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted (IB) with pAKT (Ser473), AKT1, and AKT2 (A and C) antibodies or pPKCζ/λ and PKC antibodies (B). Scanning densitometry was performed on autoradiograms from independent experiments. The results are expressed as mean ± SEM (n = 5; *$P < 0.05$ vs 5.6 mM glucose and †$P < 0.05$ vs 10 mM glucose).

samples of supernatants were collected for insulin measurement by RIA.[26]

Protein Extraction and Immunoblotting

Groups of 200 to 300 freshly isolated islets were preincubated for 30 minutes in KB at 37°C containing 5.6 mM glucose equilibrated with a mixture of 95%:5% (O_2/CO_2) and pH 7.4. This solution was then replaced, and islets were maintained for different periods in KB containing 5.6 mM glucose (G5.6), 10 mM glucose (G10), 10 mM glucose plus 0.1 mM palmitate (G10P), 10 mM glucose plus 50 μM LY294002, 10 mM glucose plus 100 nM wortmannin, or 10 mM glucose plus 0.1 mM palmitate and 15 μM fumonisin B1. Preliminary experiments were performed to establish the time course changes in the signaling proteins. The chosen periods to be studied were those in which the most pronounced changes were observed for each protein.

At the end of the incubation, the KB solution was replaced by approximately 100 μL of extraction buffer (100 mM Trizma, 1% SDS, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM ethylenediaminetetraacetic acid, and 10 mM sodium vanadate). The islets were sonicated and boiled for 10 minutes. The extracts were then centrifuged at 12,000×*g* at 4°C for 20 minutes to remove cellular debris. Protein determination of the supernatants was performed by the Bradford dye method using Bio-Rad reagent.

Equal amounts of protein from each sample were added to Laemmli sample buffer (1:5), boiled for 10 minutes, and loaded in a SDS–polyacrylamide gel. The electrotransfer of the proteins to nitrocellulose membranes was performed for 1 to 2 hours at 120 V using a Bio-Rad miniature transfer apparatus. Nonspecific protein binding to nitrocellulose was reduced by preincubating the membrane for 2 hours with blocking buffer (5% nonfat dry milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20) at room temperature. The nitrocellulose membranes were incubated for 4 hours at 22°C or overnight at 4°C with primary antibodies diluted in blocking buffer containing 3% of nonfat dry milk, and then washed for 30 minutes in blocking buffer without milk. The blots were subsequently incubated with peroxidase-conjugated secondary antibody for 1 hour and processed for chemiluminescent detection of immunoreactive bands. When necessary, the membranes were stripped, washed, and reprobed with different primary antibodies. Band intensities were quantified by optical densitometry (Scion Image-Release Beta 3b, NIH, Bethesda, MD) of the developed autoradiograph.

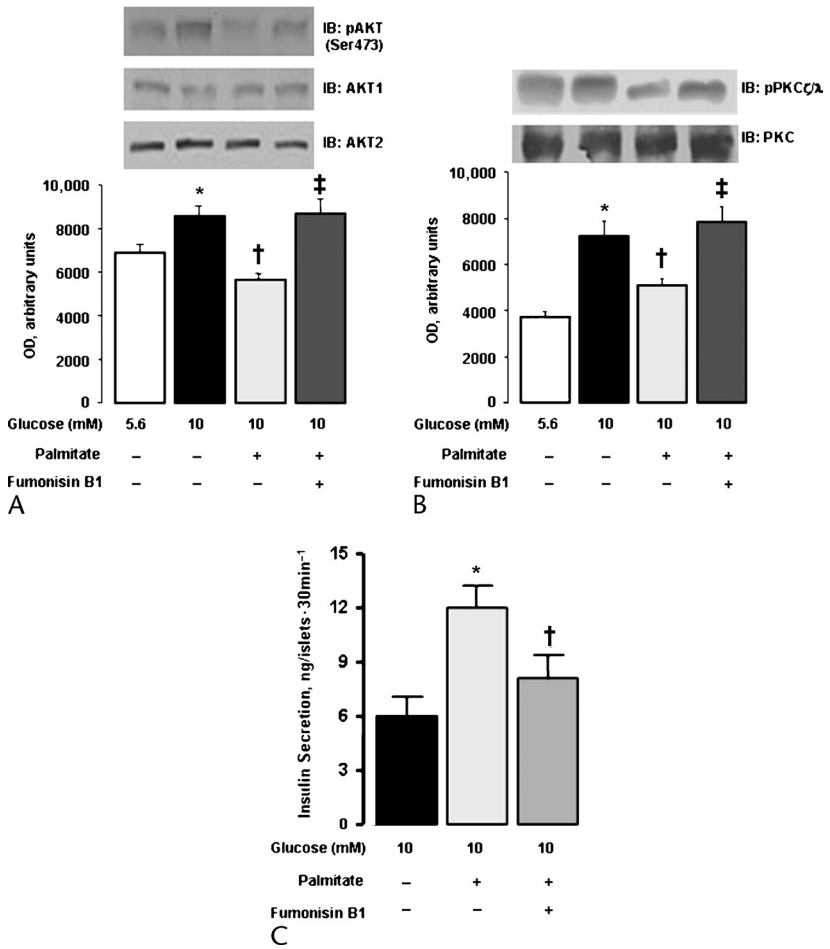

FIGURE 4. Effect of ceramide synthesis blockade on AKT (Ser473) and PKCζ/λ phosphorylation and insulin secretion. Groups of 200 to 300 freshly isolated islets were initially incubated for 30 minutes at 37°C in Krebs-Henseleit 5.6 mM glucose in the absence or presence of fumonisin B1. This solution was replaced, and islets were maintained for 5 minutes under Krebs-Henseleit containing 5.6, 10, and 10 mM glucose plus palmitate or 10 mM glucose plus palmitate and fumonisin B1. Total protein extracts (100 μg) from homogenized islets were resolved in SDS-PAGE, transferred to nitrocellulose membranes and imunnoblotted (IB) with pAKT (Ser473), AKT1, and AKT2 antibodies (n = 4) (A) or PKC and pPKCζ/λ antibodies (n = 4) (B). Scanning densitometry was performed on autoradiograms from independent experiments. Groups of 5 islets were preincubated as described in Figure 1 and after that incubated with 10 mM glucose, 10 mM glucose plus palmitate, or 10 mM glucose plus palmitate and fumonisin B1. At the end of the incubation period, samples of the supernatants were collected for insulin measurement by RIA (n = 15) (C). The results are expressed as mean ± SEM (*$P < 0.05$ vs 5.6 mM glucose, †$P < 0.05$ vs 10 mM glucose, and ‡$P < 0.05$ vs 10 mM glucose plus palmitate).

Statistical Analysis

Results are presented as mean ± SEM. Significant differences between groups were analyzed by using analysis of variance followed by the Tukey posttest. The level of significance was set at $P < 0.05$.

RESULTS

Pharmacological PI3K Inhibition and Palmitate Increase Insulin Secretion in the Presence of Stimulatory Glucose Concentration

As expected, isolated rat islets incubated for 30 minutes in the presence of 10 mM glucose (G10) displayed a 2.0-fold increase in insulin secretion when compared with G5.6. The addition of palmitate in the presence of 10 mM glucose provoked an additional 1.5-fold increase in insulin secretion. Similarly, the addition of the PI3K inhibitors (LY294002 and wortmannin) to the medium containing 10 mM glucose also provoked ~1.5-fold increase in insulin secretion. The incubation with palmitate and LY294002 in the presence of 10 mM glucose resulted in an additional increase of insulin secretion, approximately 1.5-fold higher than that observed when the drug was added alone. This additional effect was not observed with wortmannin under similar condition (Fig. 1).

Glucose-Induced Phosphorylation of AKT (Ser473) and PKCζ/λ is Blocked by Pharmacological Inhibition of PI3K and Addition of Palmitate

Glucose at 10 mM induced a 1.7-fold increase in AKT (Ser473) phosphorylation (Fig. 2A). This significant increase of AKT phosphorylation was blocked by the addition of 2 PI3K inhibitors; wortmannin and LY294002. In addition to AKT phosphorylation, 10 mM glucose induced a 1.5-fold increase in PKCζ/λ phosphorylation, which was also blocked by the addition of wortmannin and LY294002 (Fig. 2B). Similarly to wortmannin and LY294002, palmitate acutely inhibited glucose-induced AKT (Ser473) and PKCζ/λ phosphorylation (Figs. 3A, B). Incubation of pancreatic islets with 10 mM glucose and palmitate in the presence of LY294002 or wortmannin induced a further decrease of AKT (Ser473) phosphorylation (Fig. 3C).

Blockade of Ceramide Synthesis Prevents Palmitate-Stimulated Insulin Secretion and Inhibition of AKT and PKCζ/λ Phosphorylation and Insulin Secretion

Fumonisin B1 was used to investigate whether the effects of palmitate described herein were somehow secondary to ceramide generation. Fumonisin B1 treatment abolished the effect of palmitate on AKT (Ser473) and PKCζ/λ phosphorylation (Figs. 4A, B). The ceramide synthesis also abolished palmitate potentiation of insulin secretion in the presence of 10 mM glucose (Fig. 4C).

DISCUSSION

The modulation of glucose-induced insulin secretion by palmitate has been investigated by several groups.[27,28] Acute exposure of the pancreatic beta cells to both high glucose concentration and saturated FFA results in a substantial increase of insulin release, whereas a chronic exposure leads to desensitization and suppression of the secretory response.[29] The precise molecular mechanism involved in the interplay between glucose and fatty-acid metabolism and thus insulin secretion is, however, far from being completely understood. Apparently, GPR40 participates in the chronic effect of palmitate in reducing glucose-induced insulin secretion and is partially responsible for the acute FFA over the effect on insulin release in the presence of high glucose concentration.[12,30]

Glucose concentration plays a determinant role in the control of palmitate metabolism in pancreatic beta cell. To exert a stimulatory effect on insulin release, FFA need to enter the cell and conjugate with coenzyme A (CoA), forming long-chain acyl-CoAs (LC-CoA) that serve as important effector molecules, controlling a large number of signaling pathways, including PKCs and other exocytotic machinery proteins.[31,32] Under high glucose concentration, there is also an increased [U-14C]-palmitate incorporation into several phospholipids, such as phosphatidylcholine and phosphatidylserine, which are able to stimulate insulin secretion.[6]

There is consistent evidence that insulin pathway regulates its own secretion in a glucose-dependent manner.[33,34] Pancreatic beta cells express insulin receptor (IR),[35] insulin receptor substrates (IRS1/2),[36] and downstream proteins that participate in the insulin signaling pathway, such as PI3K and AKT/PKB.[37,38] Several studies have shown that PI3K participates in pancreatic insulin secretion. PI3K activation by insulin induces plasma membrane hyperpolarization that leads to a decrease in insulin secretion.[17] On the other hand, inhibition of PI3K sustains the closure of the K_{ATP} channel induced by glucose leading to a further increase of insulin secretion at high glucose concentration.[16,39] As confirmed herein, PI3K inhibition increased glucose-induced insulin secretion. LY294002 also directly inhibits the voltage-dependent K^+ current.[40,41] This additional effect may explain the more pronounced increase of insulin secretion induced by glucose in the presence of LY294002 as compared with wortmannin under similar conditions.

Glucose metabolism leads to an increase of ATP/ADP ratio and closure of ATP-sensitive K^+ channels causing membrane depolarization followed by VDCC opening and extrusion of insulin granules. Concomitantly, glucose also activates PI3K as demonstrated by others.[17] As PKCζ/λ activation occurs directly as a consequence of PI3K stimulation[42,43] or indirectly via PDK-1,[44] the activation of PI3K by glucose is corroborated by the blockade of the glucose-induced augment in AKT and PKCζ/λ phosphorylation with the addition of LY294002 and wortmannin to the incubation medium.

As PI3K activation is thought to be responsible for a decrease in insulin secretion, the present data demonstrating that stimulatory concentrations of glucose induce both AKT and PKCζ/λ phosphorylation seem apparently contradictory. However, our interpretation for these observations is that PI3K activation does not fully suppress insulin secretion

at high glucose concentrations and may serve as a constant negative feedback mechanism to prevent excessive insulin release and thus hyperinsulinemia.[17]

The involvement of PI3K in the potentiation of glucose-induced insulin secretion by palmitate was therefore investigated in the present study as a putative candidate to control pancreatic islet function. To ascertain the participation of PI3K pathway, the effect of palmitate on AKT and PKCζ/λ phosphorylation was evaluated. Palmitate mimicked the effect of LY294002 and wortmannin on the phosphorylation of these proteins, suggesting that palmitate might directly or indirectly regulates PI3K activity.

Palmitate has been shown to inhibit the PI3K/AKT pathway in insulin-responsive cells.[45,46] For instance, fatty-acid infusion (5 hours) impairs insulin-stimulated phosphorylation of AKT in skeletal muscle.[47] These effects of FFA might be a result of ceramide synthesis, a second messenger generated mainly via the de novo pathway, or activation of sphingomyelinases, which then release ceramide from sphingomyelin.[22] The sphingolipid ceramide is classically reported as a molecule that controls antiproliferative responses such as apoptosis, growth arrest, differentiation, and senescence.[48]

The decrease in AKT phosphorylation induced by palmitate is dependent on ceramide synthesis in skeletal muscle.[49,50] Stratford and collaborators demonstrated that the sphingolipid ceramide negatively regulates AKT phosphorylation by 2 independent mechanisms. First, ceramide specifically blocks the translocation of AKT to the plasma membrane through a directly binding to the pleckstrin homology domain. Second, ceramide induces the dephosphorylation of AKT by a mechanism dependent on protein phosphatase 2A.[51] However, ceramide-induced modulation of insulin signaling pathway also occurs upstream of AKT because pretreatment with C(6)-ceramide reduced insulin-induced PI3K activity in rat adipocytes.[52]

Although it has been previously suggested that inhibition of insulin secretion after chronic exposure to palmitate and oleate is probably not related to de novo ceramide synthesis,[53] this sphingolipid certainly plays an important role in pancreatic beta-cell function. Prolonged exposure to elevated levels of palmitate affects glucose-stimulated insulin gene expression, long-term insulin secretion, and cellular replication by mechanisms that involve ceramide synthesis.[21,54]

Using a pharmacological inhibitor of ceramide synthesis, fumonisin 1B, we demonstrated that the acute effect of palmitate in increasing insulin secretion and decreasing AKT and PKCζ/λ phosphorylation is probably related to ceramide synthesis by hydrolysis of sphingomyelin. This is the first study that correlates ceramide synthesis to an acute effect of palmitate in the presence of stimulatory concentrations of glucose. Similarly to our study, acute exposure to palmitate (1–2 hours) in the presence of high concentrations of glucose has been shown to decrease AKT activity due to an increase in ceramide synthesis in cardiac muscle.[46]

These results combined indicate that the modulation of PI3K pathway by palmitate occurs, at least in part, through ceramide generation. This mechanism might culminate in the down-regulation of PI3K activity (as demonstrated by AKT and PKCζ/λ phosphorylation) and an increase of insulin secretion in the presence of stimulatory concentrations of glucose. Thus, the present study describes an additional mechanism by which FFA affect pancreatic islet physiology.

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